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Abbreviations: Alas1, aminolevulinic acid synthase 1; ALT, alanine aminotransferase; APAP, acetaminophen; Arntl, aryl hydrocarbon receptor nuclear translocator-like; Arrdc3, arrestin domain containing 3; AST, aspartate aminotransferase; Atp2b2, ATPase calcium transporting, plasma membrane 2; Bmal1; brain and muscle ARNT-like 1; Clock, circadian locomotor output cycles kaput; Cry1, Cryptochrome 1; CYP, cytochromes P450; Dbp, D site albumin promoter binding protein; GEO, gene expression omnibus; GSEA, gene set enrichment analysis; H&E, hematoxylin-eosin; Mt, metallothionein; Npas2, neuronal PAS domain protein 2; Per, period circadian clock; RT-qPCR, Real-time quantitative PCR; Usp2, ubiquitin specific peptidase 2.

ABSTRACT

Drinking water can be contaminated with pharmaceuticals. However, it is uncertain whether this contamination can be harmful for the liver, especially during obesity. Hence, the goal of our study was to determine whetherchronic exposure to low doses of pharmaceuticals could have deleterious effects in livers of lean and obese mice. To this end, lean and ob/ob male mice were treated for 4 months with a mixture of 11 drugs provided in drinking water at concentrations ranging from 10 to 10^6 ng/l. At the end of the treatment, some liver and plasma abnormalities were observed in ob/ob mice treated with the cocktail containing 10^6 ng/l of each drug. For this dosage, a gene expression analysis by microarray showed altered expression of circadian genes (e.g. Bmal1, Dbp, Cry1) in lean and obese mice. RT-qPCR analyses carried out in all groups of animals confirmed that expression of 8 different circadian genes was modified in a dose-dependent manner. For some genes, a significant modification was observed for dosages as low as 10^2-10^3 ng/l. Drug mixture and obesity presented an additive effect on circadian gene expression. These data were validated in an independent study performed in female mice. Thus, our study showed that chronic exposure to trace pharmaceuticals disturbed hepatic expression of circadian genes, particularly in obese mice. Because some of the 11 drugs can be found in drinking water at such concentrations (e.g. acetaminophen, carbamazepine, ibuprofen) our data could be relevant in environmental toxicology, especially for obese individuals exposed to these contaminants.

Key words: microarray; drug; liver; mouse; obesity; circadian rhythm

Introduction

Contamination of air, soil and water with pharmaceuticals and personal care products is currently a major concern for many countries. Indeed, such contamination could endanger the health of millionsof individuals, especially in the case of chronic and multiple exposures in sensitive populations (Sanderson, 2011). Regarding pharmaceuticals, it is noteworthy that the parent drugs and their metabolites are excreted by patients as waste and some people also get rid of unused pharmaceuticals in the toilets. Hence, all these pharmaceuticals can thereafter be found in water (Kasprzyk-Hordern et al., 2008; Mompelat et al., 2009). For instance, the following drugs were detected in tap water in several independent studies: acetaminophen (APAP), bezafibrate and other fibrates, caffeine, carbamazepine, cotinine (a nicotine metabolite), diazepam, diclofenac, erythromycin, flumequine, ibuprofen, phenazone (antipyrine), roxithromycin, salicylic acid and sulfamethoxazole (Daughton and Ternes, 1999; Halling-Sorensen et al., 1998; Heberer, 2002; Mompelat et al., 2009; Stackelberg et al., 2004; Webb et al., 2003; Ye and Weinberg, 2007; Yu et al., 2007; Zuccato et al., 2000). As reported in most of these investigations, these drugs were found in the drinking water at concentrations ranging generally between 1 and 20 ng/l. However, for some pharmaceuticals such as APAP, caffeine, carbamazepine, fibrates, ibuprofen and phenazone, several hundreds of ng/l were sometimes found in tap water (Daughton and Ternes, 1999; Mompelat et al., 2009).

Long-term exposure to drug contaminants could have deleterious consequence for some sensitive tissues, especially the liver. Indeed, liver expresses high levels of cytochromes P450 (CYPs) that can transform drugs into toxic metabolites, which are able to induce oxidative stress, mitochondrial dysfunction and cell death (Aubert et al., 2012; Leung et al., 2012). It is also worthy to mention that recent investigations indicated that the liver could be particularly sensitive to drug-induced toxicity in the context of obesity (Aubert et al., 2012; Fromenty, 2013).

Taken all these data into consideration, the aim of the present study was to determine the long-term hepatic effects of pharmaceutical contaminants in lean and obese ob/ob mice. These mice were treated for 4 months with a mixture of 11 drugs provided in drinking water at concentrations ranging from 10 to 10^6 ng/l. These drugs included APAP, caffeine, carbamazepine, cotinine, diclofenac, erythromycin, ibuprofen, phenazone, roxithromycin, salicylic acid and sulfamethoxazole. Importantly, all these molecules were previously detected in drinking water, as mentioned previously.

Materials and methods

*Animals and exposure to drugs.*Five-week-old male C57BL/6J-+/+mice (wild-type, also referred to as lean mice) weighing 19 to 20g and C57BL/6J-ob/ob mice, weighing 28 to 32 g, were purchased from Janvier (Le-Genest-St-Isle, France) and housed in the animal house facility of Rennes 1 University under a 12 h light-dark cycle.All mice were fed ad libitumon a normal diet bringing 2820 kcal per kg of food (A04 biscuits; UAR,Villemoisson-sur-Orge, France). After 1 week of acclimatization,wild-type and ob/ob mice were further split into 7different groups that were treated or not with a drug cocktail containing 11 molecules at the following concentrations: 10, 10^2, 10^3, 10^4, 10^5 and 10^6 ng/l. All these drugs (APAP, caffeine, carbamazepine, cotinine, diclofenac, erythromycin, ibuprofen, phenazone, roxithromycin, salicylic acid and sulfamethoxazole) were purchased from Sigma-Aldrich (St. Quentin-Fallavier, France). Considering the molecular weight of the molecules, 10^6 ng/l corresponded to concentrations ranging from 1.2 μM for roxithromycin to 7.2 μM for salicylic acid. In a second independent series of investigations, five-week-old female C57BL/6J-+/+and C57BL/6J-ob/ob mice were purchased from Janvier (Le-Genest-St-Isle, France) and housed in the animal house facility of the Robert Debré Hospital. In this study, mice were treated or not with the same cocktail containing the 11 drugs at the following concentrations: 10^4, 10^5 and 10^6 ng/l. For both studies conducted in Rennes and Paris, mice were exposed during 4 months to these drugs by way of the drinking water, which was renewed every week. Because ob/ob mice are drinking more than lean mice (Fromenty et al. 2009; Massart et al. 2012), drug concentrations in the drinking water was adapted in the group of obese mice to keep constant the daily intake of the drugs between lean and obese mice. Consumption of water was monitored every week and it was not reduced by the presence of the mixture, whatever the drug concentrations.The last day of the treatment, blood was drawn in the morning between 10 a.m. and 2 p.m. Blood withdrawal was carried out from the retro orbital sinus with heparinized capillary Pasteur pipettes for biochemistry analyses. Mice were then sacrificed by cervical dislocation and liver was quickly removed. While a majority of the liver fragments were immediately frozen in liquid nitrogen some of them were rapidly processed for appropriate histological staining. Collected tissues frozen in liquid nitrogen were subsequently stored at -80°C until use. All experimentswere performed according to national guidelines for the useof animals in biomedical research and approved by the local Ethics Committee in Animal Experiment of Rennes 1 University and Robert Debré Hospital.

*Plasma analyses.*Immediately after collection, blood was centrifuged for 10 min at 1000g and plasma was stored at -20°C until assay. Plasma activity of alanine aminotransferase (ALT) and

aspartate aminotransferase (AST), total cholesterol, triglycerides and glucose levels were measured on an automatic analyzer AU2700 (Olympus Diagnostics, Rungis, France) with Olympus commercial Kits OSR6107, OSR6109, OSR6116, OSR6133 and OSR6121, respectively.

Liver histology. To evaluate necrosis, inflammation and steatosis, liver fragments were fixed in 10% neutral formalin and embedded in paraffin. Then, 4-µm thick sections were cut and stained with hematoxylin-eosin (H&E). All these sections were thoroughly examined by an experienced pathologist (V.T.-S.). The amount of hepatic steatosis in ob/ob and the classification of this lesion into 3 different categories (i.e. microvesicular, mediovesicular and macrovacuolar) were determined as recently described (Trak-Smayra et al., 2011).

*RNA extraction and gene expression profiling.*For the microarrays, total RNA extraction, linear T7-based amplification step, hybridization procedure, detection and read out of the fluorescence signals were carried out by Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). Total RNA was extracted from liver samples using a standard RNA extraction protocol (Trizol) and quality-checked with an Agilent 2100 Bioanalyzer platform. All RNA samples revealed RNA Integrity Number (RIN) values between 7.2 and 8. Further information on T7-based amplification, Cy3-labeling, hybridization of the Agilent microarray and detection of the fluorescent signals is given in the Supplementary Data.

Analysis and data mining of microarray data. Gene expression data were analyzed using Feature Extraction and GeneSpring softwares (Agilent Technologies) and further analyzed using R-based array tools, as previously described (Sulpice et al., 2013). Agilent Feature Extraction Software was used to read out and process the microarray image files. This software determines feature intensities (including background subtraction), rejects outliers and calculates statistical confidences. Features that were not positive and significant or below the background signal were filtered out. Microarray data were normalized by using the 75^{th} percentile shift normalization algorithm. Differentially expressed genes were identified by a two-sample univariate *t*-test and a random variance model as previously described (Coulouarn et al., 2012). Clustering analysis was done using Cluster 3.0 and TreeView 1.6 with uncentered correlation and average linkage options. Enrichment for specific biological functions or canonical pathways was evaluated by using gene ontology annotations, as described previously (Coulouarn et al., 2012). Gene set enrichment analysis (GSEA) was performed using the Java-tool developed at the Broad Institute (Cambridge, MA, USA). Microarray data were submitted to the gene expression omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo; GSE49195).

Real-time quantitative PCR (RT-qPCR) analysis. In order to study the hepatic mRNA expression of selected genes, cDNAs were preparedby reverse transcription of 1 µg of total RNA using theHigh Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Courtaboeuf, France). cDNAswere thus amplified with specific primers using the Power SYBRGreen PCR Master Mix (Applied Biosystems), in an ABI Prism 7900 instrument (Applied Biosystems). Further information on PCR analysis is provided in the Supplementary Data.

Statistical analysis. Data are presented as means ± SEM (standard error of mean). In order to assess statistical significances for body weight, plasma parameters and hepatic mRNA expression of selected genes, a two-way Analysis of variance (ANOVA) was performed with the factors of genotype (G) and drug mixture treatment (T). Individual means were compared with the post hoc Bonferroni test.

Results

Body weight, plasma parameters and liver histology

Body weight was measured on a regular basis for each mouse so that the gain of body weight could be calculated over the 4-month period of treatment. Body weight was 31.2 ± 1.0 and 60.6 ± 1.0 g, respectively in untreated lean and obese mice at the end of the treatment. The gain of body weight was higher in obese mice compared to lean mice and the treatment significantly modified this parameter (Table 1). However, it was either decreased, unchanged or increased depending in the different groups with no dose-dependent effect (Table 1).

Some plasma parameters were measured at the end of the treatment. When compared to lean mice, untreated ob/ob mice presented higher levels of plasma ALT, AST and total cholesterol but normal levels of plasma triglycerides (Table 1), as previously reported (Fromenty et al., 2009; Lindström, 2007; Massart et al., 2012). In addition, the obese mice did not present hyperglycemia (Table 1), which was consistent with previous data showing that the diabetic state of ob/ob mice ameliorated with age (Fromenty et al., 2009; Lindström, 2007). Interestingly, treatment with the drug mixtures significantly modified the plasma levels of total cholesterol and ALT (Table 1). This was particularly evident in ob/ob mice treated with the cocktail containing 10^6 ng/l of each drug since this group of animals presented the highest plasma levels of total cholesterol and ALT (Table 1).

Liver histology was also studied at the end of the treatment in mice treated or not with the cocktails containing 10^5 and 10^6 ng/l of each drug. There were no histological alterations in lean mice whatever the groups of animals (data not shown). Slight necroinflammation was observed in untreated obese mice (data not shown), which could explain higher levels of plasma AST and ALT (Begriche et al., 2008; Trak-Smayra et al., 2011). However, treatment with the drug mixture did not aggravate the histological score of necroinflammation (data not shown).

Hepatic steatosis was observed in all obese mice (Supplementary Table 1) but this lesion was absent in lean mice, treated or not with the cocktails (data not shown). Although the percentage of hepatocytes with steatosis was unchanged by the treatment in ob/ob mice, a lower proportion of microvesicular steatosis was observed in obese mice treated with the cocktail containing 10^6 ng/l of each drug (Supplementary Table 1). Accordingly, the fat droplets were larger in treated ob/ob mice when compared to the controls (Fig. 1).

Microarray analysis

Gene expression profiling was carried out in order to determine whether the cocktail containing 10^6 ng/l of each drug induced transcriptional changes in the livers of lean and obese mice. Unsupervised microarray analysis demonstrated that the cocktail significantly modified the hepatic expression of 280 and 284 genes ($P<0.05$; fold-change>1.5), respectively in lean and obese mice. Among these genes, only 21 were dysregulated in both genotypes thus suggesting that drug-induced transcriptional changes were tightly dependent of the genetic background. Table 2 provides the top 10 well-annotated genes that were up or down-regulated by the cocktail in lean and obese mice. Interestingly, when a gene ontology analysis was performed, circadian rhythm was found as the only functional category significantly enriched for the hepatic genes differentially expressed by the cocktail in both lean and obese mice (Supplementary Table 2). In addition, this analysis indicated that the functional categories were linked to biosynthetic processes and transcription factor activity in lean mice and to cell cycle and cytoskeleton for ob/ob mice (Supplementary Table 2).

Although this was not the main goal of this study, our unsupervised microarray analysis allowed us to determine the differential hepatic gene expression between lean and ob/ob mice. Our analysis identified 2,434 non-redundant genes that were differentially expressed between lean and obese mice ($P<0.001$; fold-change>2; data not shown). As previously reported (Fromenty et al., 2009; Yang et al., 2010), ob/ob mice were characterized by an increased expression of many genes involved in lipid synthesis, such as Elovl7 (x15.9), Scd2 (x12.7), Agpat9 (x8.3), Elovl6 (x7.7), Gpam (x5.3), Fasn (x4.6), Acaca (x4.6), Acly (x4.2), Pparg (x4.0) and Thrsp (x3.1). Moreover, there was in ob/ob mice a profound up- or down-regulation of the hepatic expression of several genes involved in the circadian rhythm such as Dbp (x14.3), Per3 (x7.1), Clock (x0.49), Arntl (also referred to as Bmal1) (x0.42) and Cry1 (x0.28). Interestingly, a recent study carried out in ob/ob mice reported lower hepatic expression of the last three genes compared to wild-type mice when the animals were investigated at zeitgeber times similar to our investigations (i.e. zeitgeber times 2 to 6) (Ando et al., 2011). In order to validate the whole data set obtained from our microarray analysis, a GSEA was performed using independent hepatic gene expression profiles from ob/ob and lean mice (Sharma et al. 2010), uploaded from the GEO database (GSE20878). Importantly, GSEA demonstrated that up- and down-regulated genes in ob/ob livers identified in our study were significantly enriched in the respective gene profiles of ob/ob and lean mice from the independent GSE20878 dataset (Supplementary Fig. 1).

mRNA expression of specific genes

Some genes of interest were selected to determine by RT-qPCR their hepatic expression in mice treated or not with the cocktail containing the 11 drugs at concentrations ranging from 10 to 10^6 ng/l. Since the gene ontology analysis pointed a specific dysregulation of genes involved in the

circadian rhythm in lean and obese mice, the expression of Bmal1, Clock, Dbp, Npas2, Per1, Per2, Per3, and Cry1 was studied in all groups of mice. As shown in Fig. 2, the drug cocktail presented a significant effect on gene expression, which was either decreased (Bmal1, Clock, Npas2, Cry1) or increased (Dbp, Per1, Per2, Per3). A clear dose-effect response was observed in lean and/or obese mice for the expression of all these genes. Moreover, drug mixture and obesity presented an additive effect on circadian gene expression. Importantly, the cocktail presented a significant effect on Bmal1, Dbp, Npas2, Per2, Per3, and Cry1 expression when it contained as low as 10^2 and 10^3 ng/l of each drug in the mixture (Fig. 2). For these concentrations, it could be estimated that each drug was administered to mice at the respective doses of 20 and 200 ng/kg/d, when considering the daily intake of water and the body weight of the animals.

Because our gene expression profiling was performed on male mice solely, we next validated our results on female mice. To this end, an independent series of lean and ob/ob female mice was treated or not for 4 months with mixtures containing 10^4, 10^5 and 10^6 ng/l of each drug, as described in the Methods section. Interestingly, the profiles of drug-induced modification of circadian liver gene expression were about similar to those observed in male mice, except for Per1 (Fig. 3). It was noteworthy, however, that the effect of obesity on the hepatic expression of these genes was less pronounced in female mice (Fig. 3) when compared to males (Fig. 2). We also noticed that the liver expression of the circadian genes was in general higher in female mice, especially in wild-type animals (Supplementary Fig. 2), which was consistent with a recent study (Xu et al., 2012).

Finally, we determined the hepatic expression of several genes not directly related to circadian rhythm such as Alas1, Arrdc3, Mt1, Mt2, Usp2 and Atp2b2, the expression of which was found dysregulated in the microarray analysis performed in male mice treated with the highest doses of the drugs (Table 2). Interestingly, a dose-effect response was in general observed for the expression of these 6 genes (Fig. 4). However, in female mice, only the expression of Arrdc3 and Usp2 was found to be comparable when compared to male mice (data not shown).

Discussion

This study showed for the first time that chronic oral exposure to low doses of pharmaceuticals disturbed the hepatic expression of genes involved in circadian rhythm in mice. These transcriptional effects were dose-dependent and particularly robust since they were found in lean and obese male mice but also in female animals of both genotypes. In male mice, and to a lesser extent in female mice, drug mixture and obesity presented an additive effect on the expression of circadian genes such as Dbp, Per2 and Per3. Moreover, the expression of some circadian genes was dysregulated when the drinking water contained the 11 drugs at concentrations as low as 10^2 and 10^3 ng/l. Such exposure corresponded to a daily intake of about 20 to 200 ng/kg/d. Hence, our data indicated that a mixture of pharmaceutical contaminants can alter the expression of hepatic genes at concentrations that can be found in drinking water. Indeed, for some drugs such as APAP, caffeine, carbamazepine, ibuprofen and phenazone, several hundreds of ng/l were found in tap water in previous studies (Daughton and Ternes, 1999; Mompelat et al., 2009).

A "whole mixture approach" was used in this study, and thus our investigations were not designed to determine which drug(s) could be involved in these hepatic effects, in particular on the expression of circadian genes. Nevertheless, it is noteworthy that some drugs such as prednisolone (Koyanagi et al., 2006), 5-fluorouracil (Terazono et al., 2008), interferon-α (Shinohara et al., 2008) and caffeine (Oike et al., 2011; Sherman et al., 2011) can disturb in liver the expression of genes involved in circadian rhythm. However, apart from caffeine, no information was found in the literature regarding this issue for the other drugs contained in the mixture. Although caffeine could have induced the genomic effects reported in our study, it is noteworthy that studies reporting caffeine-induced altered hepatic expression of clock genes were performed with much higher doses (from 3.5 to ca. 75 mg/kg/d) (Oike et al., 2011; Sherman et al., 2011). Thus, further investigations by treating mice with each drug separately would be needed to determine which component(s) of the mixture could alter the liver expression of clock genes when administered at very low doses. However, it is possible that the deleterious effects of the mixture uncovered in the present study might have been less significant with each individual drug (or might not have been observed), as suggested by some investigations in other experimental models (Christiansen et al., 2009; Kortenkamp et al., 2007). Interestingly, some of the drugs included in the cocktails belong to the same pharmacological classes (mainly nonsteroidal anti-inflammatory drugs and antibiotics). Therefore, it would be worth finding out whether the genomic effects detected in this study could be attributed to a particular drug class.

Although the expression of hepatic genes was disturbed in mice treated for 4 months with the cocktails containing 10^2 and 10^3 ng/l of each drug, these concentrations did not change the plasma parameters assessed in this study, namely ALT, AST, glucose, triglycerides and total cholesterol. Thus, the effects on gene expression observed in treated mice with these doses were not associated with significant hepatic cytolysis and systemic alterations of lipid homeostasis. Therefore, changes incircadian gene expression observed for these doses might not be functionally significant.It would be interesting to determine whether a much longer exposure to these low concentrations of pharmaceuticals could have harmful effects on liver biology and integrity beyond the alterations of gene expression. Furthermore, some selected circadian genes (e.g. Bmal1, Per1, Per2) could be useful markers for further investigations specifically designed to look for potential genomic effects of individual drug (or subset of drugs) included in the mixture.

It is now acknowledged that disturbances of circadian rhythmicity can promote different metabolic disorders and chronic diseases such as dyslipidemia, insulin resistance, obesity and cardiovascular diseases (Duez and Staels, 2008; Maury et al., 2010; Prasai et al., 2008). Moreover, some studies showed that liver-specific deletion of genes involved in the liver clock could disturb glucose and lipid homeostasis (Lamia et al., 2008; Tao et al., 2011). In this study, however, the genomic response to drug exposure was investigated only in the liver. Hence, further investigations would be required to determine whether an altered expression of clock genes could also be observed in other tissues such as the CNS and in tissues playing a key role in carbohydrate and lipid homeostasis. Different time points of the light-dark cycle should also be studied during these investigations.

In this study, the 4-month treatment with the cocktail containing 10^6 ng/l of each drug significantly increased plasma ALT and total cholesterol and changed the pattern of hepatic steatosis in ob/ob mice, whereas these effects were not observed in wild-type mice. For this dosage, the hepatic expression of most circadian genes in ob/ob mice was the highest, or the lowest, among the different groups of animals. Interestingly, the daily intake corresponding to this dose (ca. 0.2 mg/kg/d) was well below the pharmacological dosages for all drugs included in the mixture. Indeed, the daily dosage of these drugs is comprised between a few mg/kg/d for diclofenac and roxithromycin to 40 and 60 mg/kg/d for salicylic acid and APAP, respectively. Importantly, some of these drugs such as APAP, diclofenac, ibuprofen and salicylate can be taken over the long-term (i.e. several months) for the treatment of inflammatory diseases or chronic pain (Kuffner et al., 2006; Schaffer et al., 2006), which are often observed in obese patients (Guh et al. 2009; Wright et al. 2010). Thus, our work could also be relevant for obese patients with multiple drug prescriptions, since obesity appears to increase the risk of hepatotoxicity, at least for some drugs (Fromenty, 2013; Massart et al., 2012). Further investigations will be needed to determine whether the genomics

changes observed in this study could be involved in higher plasma ALT and total cholesterol observed in ob/ob mice.

In conclusion, our study showed for the first time that chronic exposure to trace pharmaceuticals was able to disturb the hepatic expression of genes involved in circadian rhythm in lean and obese mice. This effect was dose-dependent and the expression of some circadian genes was already dysregulated when the drinking water contained the 11 drugs at concentrations as low as 10^2 and 10^3 ng/l. Moreover, drug mixture and obesity presented an additive effect on this gene expression. Because some of the 11 drugs can be found in the drinking water at such concentrations (e.g. APAP, caffeine, carbamazepine, ibuprofen, phenazone) further investigations would be needed to determine whether our data could be extrapolated to human populations exposed to pharmaceutical contaminants, in particular in countries with high prevalence of obesity.

Acknowledgments

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Supplementary data

Supplemental data were provided with this manuscript. The transcriptomic dataset (GSE49195) presented in this study is available in the GEO database at the following URL address: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=jvgblqimewcyaju&acc=GSE49195.

Conflict of interest

Sébastien Anthérieu, Dounia Menouer, Cédric Coulouarn, Karima Begriche, Viviane Trak-Smayra, Sophie Martinais and Marie-Anne Robin have nothing to disclose. Mathieu Porceddu reports co-founding of Mitologics SAS, outside the submitted work. Bernard Fromenty reports

personal fees from Medicines for Malaria Venture and from Medicen Paris Region, and a grant from Société Francophone du Diabète, outside the submitted work.

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Table 1.

Body weight gain and plasma parameters in the different groups of mice at the end of the treatment.

Animals	**Treatment**	**Body weight gain (g)**	**Glucose (mM)**	**Total cholesterol (mM)**	**Triglycerides (mM)**	**ALAT (UI/L)**	**ASAT (UI/L)**
Lean	0	11.1 ± 0.8	9.6 ± 0.9	2.49 ± 0.12	1.42 ± 0.17	86 ± 15	194 ± 50
	10 ng/l	9.8 ± 2.1	9.2 ± 0.6	2.21 ± 0.09	1.00 ± 0.09	90 ± 13	147 ± 25
	10^2 ng/l	11.4 ± 0.7	8.8 ± 0.4	2.28 ± 0.08	1.21 ± 0.16	76 ± 9	164 ± 30
	10^3 ng/l	10.5 ± 0.4	9.4 ± 0.4	2.31 ± 0.09	1.15 ± 0.08	113 ± 26	187 ± 24
	10^4 ng/l	11.9 ± 0.5	10.0 ± 0.4	2.43 ± 0.05	1.29 ± 0.10	71 ± 5	145 ± 23
	10^5 ng/l	12.8 ± 0.3	10.2 ± 0.6	2.67 ± 0.08	1.34 ± 0.03	147 ± 27	189 ± 14
	10^6 ng/l	11.3 ± 0.6	9.9 ± 0.5	2.53 ± 0.09	1.35 ± 0.18	101 ± 13	159 ± 24
ob/ob	0	30.4 ± 0.8	7.2 ± 0.7	7.88 ± 0.38	1.27 ± 0.12	708 ± 50	451 ± 24
	10 ng/l	27.7 ± 1.5	10.2 ± 3.1	7.43 ± 0.65	1.12 ± 0.09	672 ± 62	674 ± 105
	10^2 ng/l	31.7 ± 1.6	8.1 ± 0.9	7.81 ± 0.29	1.06 ± 0.02	674 ± 75	587 ± 85
	10^3 ng/l	30.0 ± 0.6	9.5 ± 1.2	7.73 ± 0.44	1.16 ± 0.08	533 ± 58†	372 ± 42
	10^4 ng/l	31.3 ± 0.9	8.9 ± 0.7	8.16 ± 0.32	1.19 ± 0.08	683 ± 43	748 ± 230
	10^5 ng/l	33.3 ± 0.5	9.8 ± 1.0	8.46 ± 0.29	1.07 ± 0.03	844 ± 104	759 ± 133
	10^6 ng/l	29.1 ± 0.9	9.4 ± 0.5	8.83 ± 0.28†	1.29 ± 0.05	936 ± 56†	702 ± 76
Two-way ANOVA analysis		**G,T**		**G,T**		**G,T,GxT**	**G**

Note: Plasma parameters were determined in the fed state. Body weight gain was calculated for each mouse over the 4 months of treatment. Results are mean ± SEM for 5 to 7 mice. Statistical analysis was performed with a two-way ANOVA: G, effect of genotype, T, effect of treatment, GxT, interaction between genotype and treatment. Individual means were then compared with the *post hoc* Bonferroni test. †Significantly different from untreated mice of the same genotype ($P<0.05$).

Table 2.

Top 10 well-annotated genes that were up or down-regulated in liver of wild-type and ob/ob mice treated with the cocktail containing 10^6 ng/l of each drug.

Up-regulated in both lean and ob/ob mice[a]	**Name (Accession number)**	**Gene ontology**[b]
Dbp (lean 3.70 ; ob 2.08)[c]	D site albumin promoter binding protein (NM_016974)	Circadian rhythm. Positive regulation of transcription from RNA polymerase II promoter
Alas1 (lean 2.56 ; ob 1.52)	Aminolevulinic acid synthase 1 (NM_020559)	Biosynthetic process. Heme biosynthetic process
Ypel1 (lean 1.89 ; ob 1.54)	Yippee-like 1 (Drosophila) (NM_023249)	Negative regulation of protein kinase B signaling cascade. Regulation of myelination
Gnat1 (lean 1.79 ; ob 1.79)	Guanine nucleotide binding protein, alpha transducing 1 (NM_008140)	Adenylate cyclase-modulating G-protein coupled receptor signaling pathway. Cell proliferation
Pfkfb3 (lean 1.79 ; ob 1.59)	6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (NM_133232)	Fructose 2,6-biphosphate metabolic process. Phosphorylation
Arrdc3 (lean 1.79 ; ob 1.52)	Arrestin domain containing 3 (NM_001042591)	Fat pad development. Negative regulation of heat generation
Down-regulated in both lean and ob/ob mice	**Name (Accession number)**	**Gene ontology**
Cry1 (lean 0.38 ; ob 0.65)	Cryptochrome 1 (photolyase-like) (NM_007771)	Circadian rhythm. DNA repair
Hmgcs1 (lean 0.45 ; ob 0.61)	3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (AK078743)	Cholesterol biosynthetic process. Isoprenoid biosynthetic process
Trim27 (lean 0.45 ; ob 0.63)	Tripartite motif-containing 27 (AK049314)	Interferon gamma secretion. Negative regulation of adaptive immune response
Cstad (lean 0.50 ; ob 0.63)	CSA-conditional, T cell activation-dependent protein (NM_030137)	Mitochondrial membrane organization
Avpr1a (lean 0.54 ; ob 0.67)	Arginine vasopressin receptor 1A (NM_016847)	Calcium-mediated signaling. Elevation of cytosolic calcium ion concentration
Lonrf3 (lean 0.56 ; ob 0.58)	LON peptidase N-terminal domain and ring finger 3 (NM_028894)	Proteolysis
Cdca2 (lean 0.56 ; ob 0.61)	Cell division cycle associated 2 (NM_175384)	Cell cycle. Cell division
Arntl (lean 0.57 ; ob 0.36)	Aryl hydrocarbon receptor nuclear translocator-like (NM_007489)	Circadian regulation of gene expression. Circadian rhythm
Chka (lean 0.60 ; ob 0.56)	Choline kinase alpha (NM_013490)	CDP-choline pathway. Glycerophospholipid biosynthetic process
Cxadr (lean 0.62 ; ob 0.56)	Coxsackie virus and adenovirus receptor (NM_009988)	Actin cytoskeleton reorganization. AV node cell to bundle of His cell communication
Up-regulated only in lean mice	**Name**	**Gene ontology**
Cfd (4.35)	Complement factor D (adipsin) (NM_013459)	Complement activation, alternative pathway. Innate immune response
H19 (3.85)	H19 fetal liver mRNA (AK003142)	Negative regulation of cell proliferation. Regulation of gene expression
Tppp3 (2.94)	Tubulin polymerization-promoting protein family member 3 (NM_026481)	Microtubule bundle formation
Inhbb (2.63)	Inhibin beta-B (NM_008381)	Activin receptor signaling pathway. Cellular response to cholesterol
Kif23 (2.50)	Kinesin family member 23 (NM_024245)	Spindle midzone assembly involved in mitosis
E2f7 (2.44)	E2F transcription factor 7 (NM_178609)	Cell cycle. Chorionic trophoblast cell differentiation
Pdlim3 (2.44)	PDZ and LIM domain 3 (NM_016798)	Actin filament organization. Heart development
Igfbp6 (2.38)	Insulin-like growth factor binding protein 6 (X81584)	Regulation of cell growth
Esm1 (2.33)	Endothelial cell-specific molecule 1 (NM_023612)	Biological process. Regulation of cell growth
Il1rap (2.33)	Interleukin 1 receptor accessory protein (NM_008364)	Cytokine-mediated signaling pathway. Innate immune response
Down-regulated only in lean mice	**Name**	**Gene ontology**
Fos (0.19)[2]	FBJ osteosarcoma oncogene (NM_010234)	Cellular response to cellular calcium ion. Cellular response to extracellular stimulus.
Nr4a1 (0.21)	Nuclear receptor subfamily 4, group A, member 1 (NM_010444)	Apoptotic process. Cell migration involved in sprouting angiogenesis
Egr1 (0.31)	Early growth response 1 (NM_007913)	Bone morphogenetic protein signaling pathway. Cellular

		response to gamma radiation
Dusp1 (0.36)	Dual specificity phosphatase 1 (NM_013642)	Cell cycle. Dephosphorylation
Igh-VJ558 (0.40)	Immunoglobulin heavy chain (J558 family) (XM_001474025)	
Igfbp1 (0.40)	Insulin-like growth factor binding protein 1 (NM_008341)	Regulation of cell growth
Fam65b (0.42)	Family with sequence similarity 65, member B (NM_029679)	Biological process
Zfp36 (0.44)	Zinc finger protein 36 (NM_011756)	3'-UTR-mediated mRNA stabilization. Intracellular protein kinase cascade
Fam110c (0.45)	Family with sequence similarity 110, member C (NM_027828)	Positive regulation of cell migration. Positive regulation of protein kinase B signaling cascade
Slc34a2 (0.46)	Solute carrier family 34 (sodium phosphate), member 2 (NM_011402)	Cellular phosphate ion homeostasis. In utero embryonic development
Up-regulated only in ob/ob mice	**Name**	**Gene ontology**
Clec2d (2.86)	C-type lectin domain family 2, member d (NM_053109)	Cellular defense response. Negative regulation of osteoclast differentiation
Usp2 (2.78)	Ubiquitin specific peptidase (NM_198092)	Cell cycle. Muscle organ development
Mt2 (2.63)	Metallothionein 2 (NM_008630)	Cellular response to drug. Cellular zinc ion homeostasis
Upp2 (2.56)	Uridine phosphorylase 2 (NM_029692)	Metabolic process. Nucleoside metabolic process
Mt1 (2.44)	Metallothionein 1 (NM_013602)	Cellular metal ion homeostasis. Cellular response to chromate
Krt20 (2.33)	Keratin 20 (NM_023256)	Apoptotic process. Cellular response to stress
Rgs16 (2.27)	Regulator of G-protein signaling 16 (NM_011267)	G-Protein coupled receptor signaling pathway. Negative regulation of signal transduction
Car3 (2.08)	Carbonic anhydrase 3 (AK075630)	One-carbon metabolic process. Response to oxidative stress
Grm8 (1.92)	Glutamate receptor, metabotropic 8 (NM_008174)	Adenylate cyclase inhibiting G-protein coupled glutamate receptor signaling pathway. G-protein coupled receptor signaling pathway
Cdc6 (1.85)	Cell division cycle 6 homolog (S. cerevisiae) (NM_011799)	Cell cycle. Cell division
Down-regulated only in ob/ob mice	**Name**	**Gene ontology**
Moxd1 (0.18)	Monooxygenase, DBH-like 1 (NM_021509)	Catecholamine metabolic process. Oxidation-reduction process
Hsd3b5 (0.18)	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 5 (NM_008295)	Oxidation-reduction process. Steroid biosynthetic process
Man2a2 (0.40)	Mannosidase 2, alpha 2 (NM_172903)	Carbohydrate metabolic process. Mannose metabolic process
Usp26 (0.45)	Ubiquitin specific peptidase 26 (NM_031388)	Protein deubiquitination. Proteolysis
Gadd45g (0.45)	Growth arrest and DNA-damage-inducible 45 gamma (NM_011817)	Activation of MAPKK activity. Apoptotic process
Atp2b2 (0.45)	ATPase, Ca2+ transporting, plasma membrane 2 (NM_009723)	ATP catabolic process. Calcium ion export
Ppp1r9a (0.46)	Protein phosphatase 1, regulatory (inhibitor) (NM_181595)	Actin filament organization. Calcium-mediated signaling
Kalrn (0.47)	Kalirin, RhoGEF kinase (AK051053)	Axonogenesis. Intracellular signal transduction
Murc (0.48)	Muscle-related coiled-coil protein (NM_026509)	Cardiac myofibril assembly. Cell differentiation
Elovl3 (0.48)	Elongation of very long chain fatty acids-like 3 (NM_007703)	Fatty acid biosynthetic process. Fatty acid elongation, monounsaturated fatty acid

Note:[a]Less than 10 genes were found to be modulated by the drug mixture in this category. [b]The first 2 terms were selected by using the Amigo Gene Ontology website, with "*Mus musculus*" and "*Biological process*" as filters. [c]The number in parentheses is the fold change given by the microarray analysis.

Legends to the figures

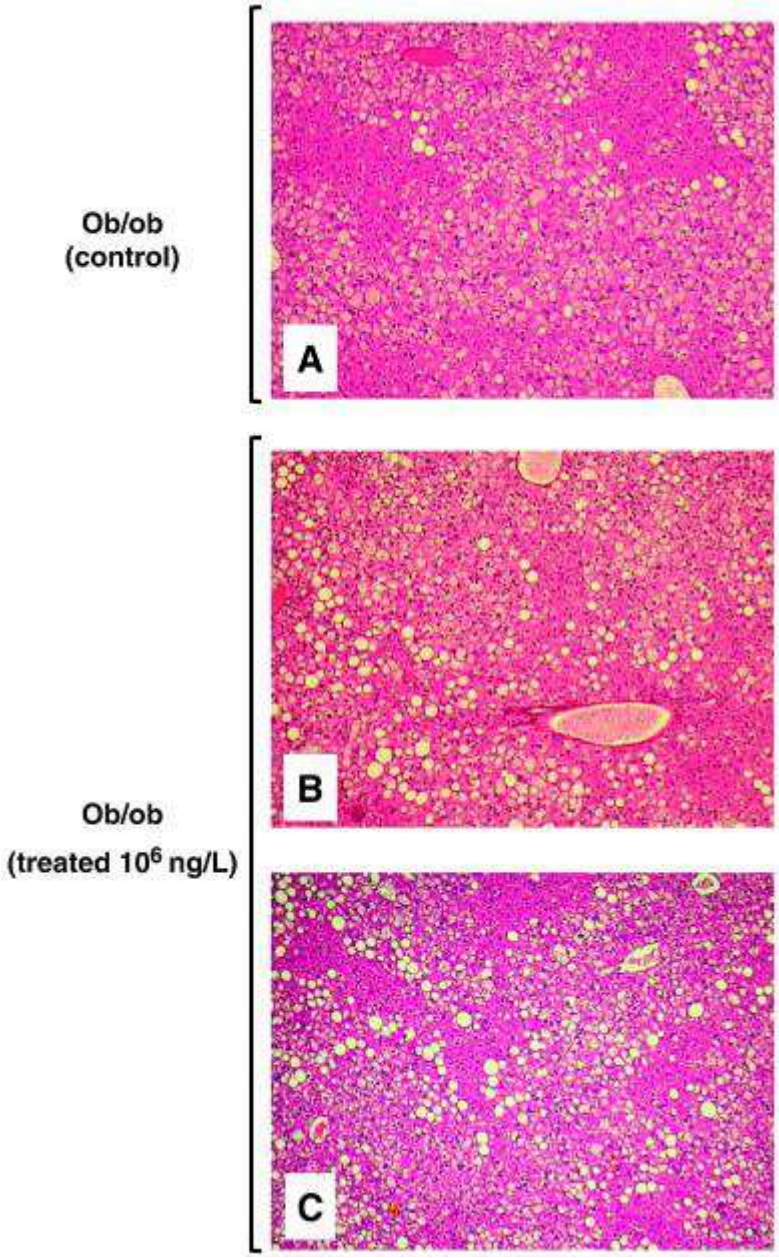
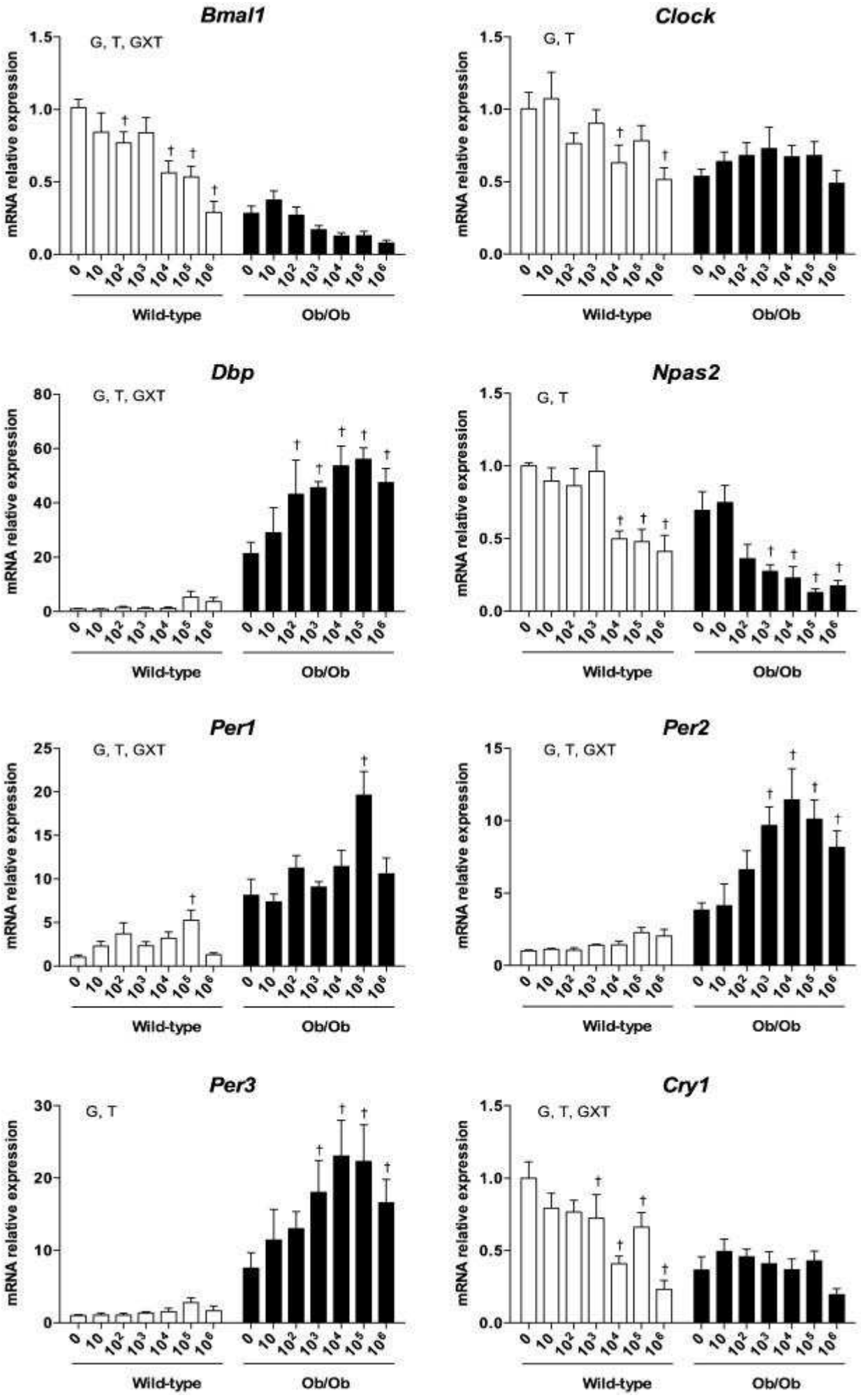
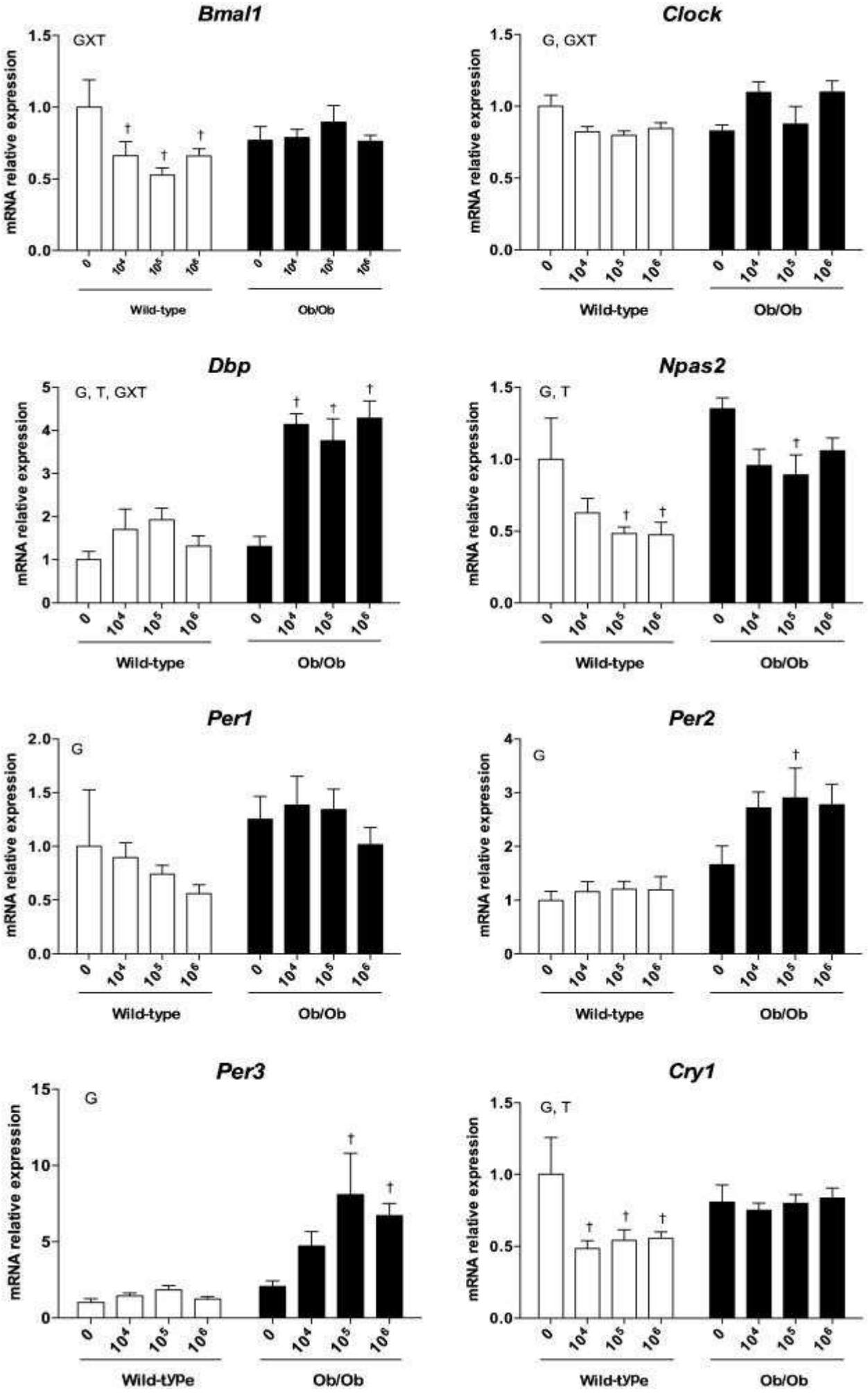


Figure 1. Hepatic steatosis in obese ob/ob mice treated or not with the cocktail containing 10^6 ng/l of each drug. Liver sections were stained with H&E and pictures were taken at 200x magnification. Mice presented in panels A, B and C are those numbered 1, 14 and 18 in Supplementary Table 1.


Bmal1
G, T, GXT
mRNA relative expression
Wild-type
Ob/Ob
Clock
G, T
Dbp
Npas2
Per1
Per2
Per3
Cry1

527

Figure 2. Hepatic mRNA expression of 8 circadian genes in male lean and obese mice treated or not with the cocktail containing the 11 drugs at concentrations ranging from 10 to 10^6 ng/l. Results are mean ± SEM for 5 to 7 mice. Statistical analysis was performed with a two-way ANOVA: G, effect of genotype, T, effect of treatment, GxT, interaction between genotype and treatment. Individual means were then compared with the *post hoc* Bonferroni test. †Significantly different from untreated mice of the same genotype ($P<0.05$).


Bmal1
GXT
mRNA relative expression
1.5
1.0
0.5
0.0
†
†
†
0
10⁴
10⁵
10⁶
Wild-type
Ob/Ob
Clock
G, GXT
Dbp
G, T, GXT
5
4
3
2
1
Npas2
G, T
Per1
G
2.0
Per2
Per3
15
10
5
Cry1

535

Figure 3. Hepatic mRNA expression of 8 circadian genes in female lean and obese mice treated or not with the cocktail containing the 11 drugs at concentrations ranging from 10^4 to 10^6 ng/l. Results are mean ± SEM for 4 to 8 mice. Statistical analysis was performed with a two-way ANOVA: G, effect of genotype, T, effect of treatment, GxT, interaction between genotype and treatment. Individual means were then compared with the *post hoc* Bonferroni test. †Significantly different from untreated mice of the same genotype ($P<0.05$).

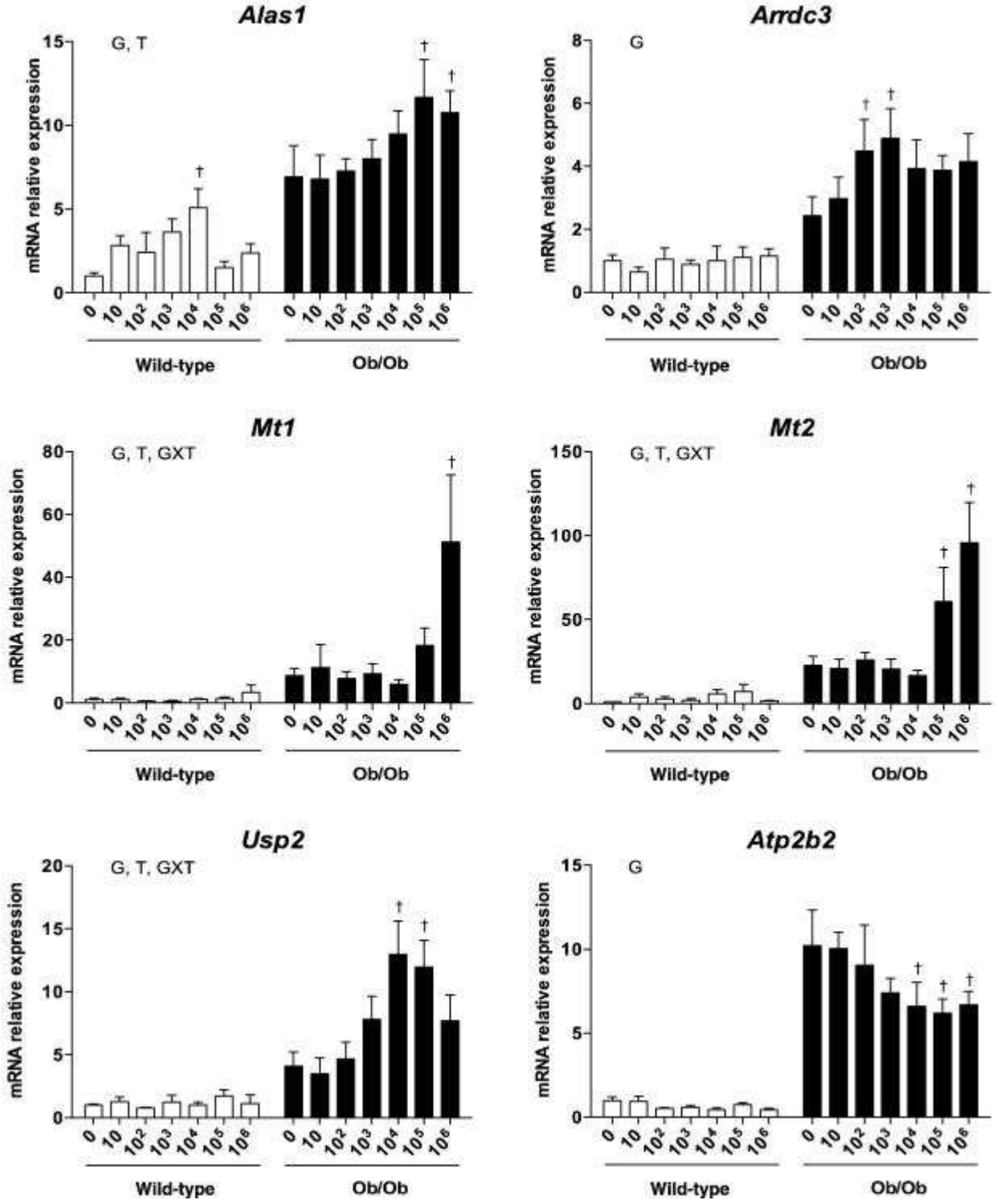


Figure 4. Hepatic mRNA expression of 6 non-circadian genes in male lean and obese mice treated or not with the cocktail containing the 11 drugs at concentrations ranging from 10 to 10^6 ng/l. Results are mean ± SEM for 5 to 7 mice. Statistical analysis was performed with a two-way ANOVA: G, effect of genotype, T, effect of treatment, GxT, interaction between genotype and treatment. Individual means were then compared with the *post hoc* Bonferroni test. †Significantly different from untreated mice of the same genotype (P<0.05).